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Colistin-Oxolinic Acid-Blood Agar: a New Selective Medium for Streptococci

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The development and evaluation of a new selective medium (colistin-oxolinic acid-blood agar) for streptococci is described. Streptococci of medical and veterinary importance grew well on the medium. Gram-negative organisms, staphylococci, *Bacillus* spp., and coryneforms are all inhibited. It was concluded that the medium is valuable for the isolation of streptococci in pure culture from mixed flora and has advantages over other media previously described. Increased isolation rates were obtained together with earlier identification of the isolated strains.

The isolation of streptococci is often complicated by the presence of other bacterial flora, which may either overgrow or restrict the growth of the streptococci. A number of media selective for streptococci have been described (1, 2, 5–7, 9, 10, 13, 14, 18, 19). In certain of these media, some of the selective agents have an adverse effect on some species of streptococci (3, 5, 6, 9, 11, 12, 14–16), including those for which the medium was developed.

During the development of a selective broth medium for the rapid detection of streptococci (Petts, M.S. thesis, University of Surrey, Guildford, Surrey, England, 1981), it was noted that when very small numbers of *Streptococcus pyogenes* cells are used to inoculate broths containing concentrations of gentamicin, amikacin, and fucidic acid below the minimum inhibitory concentrations of these antibiotics for this organism, the growth of *S. pyogenes* is inhibited. When large inocula are used, normal growth is obtained. Agents which allow good growth from very small inocula and show no effect on *S. pyogenes* are colistin, nalidixic acid, and oxolinic acid. Colistin and nalidixic acid in combination inhibit gram-negative organisms but not staphylococci and coryneforms. The inhibition of staphylococci, coryneforms, and gram-negative organisms is achieved by replacing nalidixic acid with oxolinic acid. A combination of colistin and oxolinic acid in Todd-Hewitt broth allows good growth of a wide range of *Streptococcus* spp., but inhibits the growth of *Enterobacteriaceae*, *Pseudomonas aeruginosa*, staphylococci, and coryneforms. In view of this, it was decided to investigate whether colistin and oxolinic acid could be used in blood agar as selective agents for the isolation of streptococci.

MATERIALS AND METHODS

Development of the selective combination. The streptococcal strains used in this part of the investigation are shown in Table 1. With the exceptions noted, all of the human strains were recent clinical isolates. These were identified by either Streptex (Wellcome Diagnostics, Temple Hill, Dartford, England) or the API 20 Strep (API Laboratory Products Ltd., Basingstoke, England). The animal strains were also recent isolates. The strains of *S. equi* and equine isolates of *S. equisimilis* were received from J. G. Atherton of the Equine Research Station, Newmarket, England. The bovine strains of *S. zooepidemicus*, *S. dysgalactiae*, *S. uberis*, and *S. agalactiae* were received from P. G. Francis of the Central Veterinary Laboratory, Newhaw, England. The porcine isolates of group L and *S. suis* type 1 were received from G. J. Dagnell of the Department of Animal Husbandry and Hygiene, Royal Veterinary College, Potters Bar, England. The isolate of *S. suis* type II was received from J. Nelson of the Veterinary Investigation Centre, Cambridge, England. Strains of *S. bovis* types I and II, *S. durans*, *S. faecalis*, and *S. faecium* were received from M. B. McIllmurray of Wellcome Research Laboratories, Beckenham, England.

Colistin sulfate and oxolinic acid were added to Columbia agar (Oxoid CM33) with 5% defibrinated horse blood (COBA medium) to give combined concentrations of 10 mg of colistin per liter with oxolinic acid concentrations of 5, 7.5, 10, 15, and 20 mg/liter. Todd-Hewitt broth (Oxoid CM189) cultures of the streptococci shown in Table 1 were used to perform colony counts on the range of colistin and oxolinic acid combinations. Dilutions were prepared in sterile distilled water from 10^{-1} to 10^{-8}, and 3 volumes of 10 μl each were pipetted onto the agar. When dry, the plates were incubated at 37°C overnight in an atmosphere enriched with 5% carbon dioxide. Columbia blood agar with 5% defibrinated horse blood was used as the control medium.

The ability of the combination to prevent the growth of organisms other than streptococci while allowing streptococci to grow was evaluated with 66 clinical swabs known to contain the following wild-type clinical isolates in various combinations: *P. aeruginosa*, coagulase-negative staphylococci, *Staphylococcus aureus*, *Proteus* spp., *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., streptococci of groups A, B, C, and G, *S. pneumoniae*, and viridans streptococci. These were inoculated onto blood agar and COBA medium containing 10 mg of colistin per liter and oxolinic acid at 5, 7.5, and 10 mg/liter. These combinations were also inoculated with 2-μl volumes of overnight broth cultures of five wild strains each of *Proteus* spp., *P. aeruginosa*, *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Providencia stuartii*, *S. aureus*, and coagulase-negative staphylococci. Each of these inocula contained between 10^4 and 10^5 CFU.

Colistin (10 mg/liter) and oxolinic acid (5 mg/liter) were added to Islam medium (8), which was then inoculated with four beta-hemolytic strains of *S. agalactiae*. After anaerobic 5% CO_2 incubation at 37°C, pigmentation of the *S. agalactiae* colonies was compared with that obtained on nonselective Islam medium similarly inoculated.

To determine whether the combination of 10 mg of colistin

TABLE 1. Mean colony counts of *Streptococcus* spp. on Columbia blood agar containing various combinations of colistin and oxolinic acid

Species	Lancefield group	Source	No. of strains	Mean CFU/cm³ (10^6) obtained from broth cultures on[a]:					
				BA	BA + COL-10 + OX-5	BA + COL-10 + OX-7.5	BA + COL-10 + OX-10	BA + COL-10 + OX-15	BA + COL-10 + OX-20
S. pyogenes	A	Human	10	31.5	44.6	37.8	25.3[b]	25.1[b]	10.5[b]
S. agalactiae	B	Human	10	27.1	28.5	27.3	30.8	27.9	25.0
		Bovine	2						
S. dysgalactiae	C	Bovine	1	80	65	55	35[b]	NT[c]	NT
S. equi	C	Equine	2	52	48	53	47[b]	NT	NT
S. equisimilis	C	Human	9	35	20.5	23.1	32.7	23[b]	29[b]
S. zooepidemicus	C	Bovine	1	18.2	21.4	11.8	11.0	NT	NT
		Equine	2						
S. faecalis	D	Human	18	83.1	50.5	61.7[b]	NG[d]	NT	NT
		Bovine	3						
S. faecium	D	Human	5	20	21.5	19.6	21[b]	NT	NT
S. durans	D	Bovine	2	54	65	70	50	NT	NT
S. bovis	D	Bovine	2	52	63	62	45[b]	NT	NT
S. uberis	E	Bovine	1	21	15	20	14[b]	NT	NT
S. milleri	F	Human	1	90	100	30	100	NT	NT
"Large colony"	G	Human	10	85.8	90.9	89.5	45[b]	32.8[b]	NT
S. salivarius	K	NCTC 8606	1	40	35	25	29	NT	NT
Unnamed	L	Porcine	2	27	23	29	28	NT	NT
S. suis II	R	Porcine	1	47	50	53	10	NT	NT
S. suis I	S	Porcine	1	25	24	26	15	NT	NT
S. mitis		Human	1	50	54	60	57	NT	NT
S. mutans		NCTC 10449	1	100	80	20[b]	NG	NT	NT
S. pneumoniae		Human	5	27	25.4	23.2	29.1	NT	NT
S. sanguis		Human	1	40	35	25	29[b]	NT	NT

[a] BA, Columbia blood agar; COL-10, colistin (10 mg/liter); OX-5, -7.5, -10, -15, and -20, oxolinic acid (5, 7.5, 10, 15, and 20 mg/liter, respectively).
[b] Reduction in colony size or hemolysis or both.
[c] NT, Not tested.
[d] NG, No growth.

and 5 mg of oxolinic acid per liter would allow streptococci to grow on blood agar made with bases other than Columbia, these compounds were added to tryptose-blood agar base (Oxoid CM233), tryptone-soya agar (Oxoid CM131), blood agar base (Oxoid CM55), and blood agar base no. 2. (Oxoid CM271), each containing 5% defibrinated horse blood.

These plates were inoculated with broth cultures of each of the species of streptococci shown in Table 1. The growth obtained was compared with the growth on the equivalent blood agar and with Columbia blood agar with and without the selective agents. These media were also inoculated with swabs known to contain *Proteus* spp., *P. aeruginosa*, *Staphylococcus* spp., coliforms, streptococci of groups A, B, C, D, and G, and *S. pneumoniae*.

Evaluation of the medium. The ability of CBA medium containing 10 mg of colistin and 5 mg of oxolinic acid per liter to grow streptococci from clinical specimens was investigated by using 462 swabs from skin, ear, and nose infections. The isolation of streptococci and the growth of other organisms on COBA medium were compared on Columbia blood agar and Columbia blood agar containing 10 mg of colistin and 10 mg of nalidixic acid per liter (CNA medium) (4). The streptococci isolated and identified in this trial were identified by Streptex, by optochin sensitivity for *S. pneumoniae*, or by the API 20 Strep as appropriate. Other organisms isolated were identified by standard methods.

RESULTS

The results shown in Table 1 indicate that concentrations of oxolinic acid up to 10 mg/liter combined with 10 mg of colistin per liter had no serious effect on the viable counts of the streptococci tested. The exceptions to this were *S. faecalis* and *S. mutans*, which failed to grow well on oxolinic acid concentrations higher than 7.5 mg/liter. Several other species showed a reduction in colony size or degree of hemolysis at 10 mg of oxolinic acid per liter (Table 1).

None of the 15 *P. aeruginosa*, 10 *Proteus* spp., 13 coliform, 39 *Staphylococcus* spp., or 3 coryneform strains present in the 66 laboratory-inoculated swabs grew on any of the selective combinations. All of the 19 group A, 7 group B, 8 group C, 18 group G, 6 *S. pneumoniae*, and 4 viridans streptococcal strains grew well on all combinations. There was a slight reduction of hemolysis with three strains of group A and three strains of group C streptococci in the medium containing 10 mg of oxolinic acid per liter. All of the 16 group D streptococci isolated grew on the medium containing 5 mg of oxolinic acid per liter, 10 grew on 7.5 mg of oxolinic acid per liter, and 6 grew on 10 mg of oxolinic acid per liter. Those which grew on the highest concentration were *S. faecium* strains. The *S. faecalis* strains isolated failed to grow on the highest concentration, and those which grew at 7.5 mg of oxolinic acid per liter produced smaller colonies. There was no real difference in the colonial appearance of *S. faecalis* on the medium containing 5 mg of oxolinic acid per liter.

All of the combinations of colistin and oxolinic acid investigated prevented the growth of the wild strains of nonstreptococcal organisms inoculated from broth cultures.

These results indicated that a combination of 10 mg of colistin and 5 mg of oxolinic acid per liter would allow

growth of the streptococcal species tested. This combination also inhibited staphylococci, coryneforms, coliforms, *Proteus* spp., and *P. aeruginosa* and was selected for further testing.

It was found that the selected combination did not affect the pigmentation of group B streptococci on Islam medium. This combination was also selective for streptococci when incorporated into tryptose, tryptone soya, blood agar base, and blood agar base no. 2. blood agars. The colony size and quality of the growth on these media (both selective and nonselective) were inferior to those obtained on Columbia blood agar.

The results obtained from the 462 swabs used in the clinical trial of COBA medium are summarized in Tables 2 and 3. A total of 216 streptococci were isolated from 190 swabs. Pure growths of streptococci were obtained from 185 of the swabs when plated onto COBA medium. Colonies of other organisms were not visible, and when subcultures of the streptococci were made from colonies growing on COBA medium, pure growths were obtained. Gram-negative organisms (122 coliforms, 61 *Proteus* spp., 59 *P. aeruginosa*, and 1 *Haemophilus* sp.) did not grow on COBA or CNA medium. Gram-positive organisms other than streptococci (252 *Staphylococcus* spp., 57 coryneforms, and 7 *Bacillus* sp.) were inhibited on COBA medium. A total of three coagulase-negative staphylococci and two coryneforms grew on COBA medium, but the number of colonies was very reduced when compared with the growth on Columbia blood agar or CNA medium. A total of 25 swabs grew two types of streptococci on COBA and CNA media; on Columbia blood agar only, 16 swabs grew two types of streptococci. These swabs were all from skin lesions, and the combinations and the number of isolates on COBA and CNA media were (the corresponding values for Columbia blood agar are shown in parentheses): groups A and G, 1 (0); group A and *S. pneumoniae*, 1 (1); group A and nonhemolytic streptococci, 1 (1); groups B and C, 1 (1); groups B and D, 1 (1); groups B and G, 2 (1); group B and nonhemolytic streptococci, 4 (2); groups C and D, 7 (4); groups D and F, 4 (4); group D and nonhemolytic streptococci, 1 (1); group D and *S. milleri*, 1 (0); group G and *S. pneumoniae*, 1 (0).

DISCUSSION

A comparison of colony counts obtained on an inhibitory medium with those obtained on a noninhibitory medium is widely used for assessing the suppressive qualities of a selective medium. With streptococci, the colony count does not equate to the number of cells in a broth but does to the number of colony-forming chains. Variation in the length of streptococcal chains and in colony-counting methods makes statistical analysis of the result difficult. Nevertheless, the results show no real difference between the colony counts on Columbia blood agar and COBA medium. This result and the results of the clincial trial indicate that COBA medium is not inhibitory to the streptococci investigated. The range of species investigated includes many which are important human and animal pathogens. Preliminary experiments undertaken since the completion of this investigation show that peptococci and peptostreptococci also grow well on the medium.

TABLE 2. Comparison of streptococci isolated from swabs on Columbia blood agar, COBA, and CNA

Blood agar used	No. of isolates[a]								
	A	B	C	D	G	*S. pneumoniae*	*S. milleri*	Others[b]	Total (% of total)
Columbia	13	12	13	41	24	7	3	29	142 (65)
CNA	18	23	16	71	32	8	3	40	211 (97.6)
COBA	19	24	17	70	32	8	3	42	215 (99.5)

[a] A to G, Lancefield groups.
[b] Viridans type and nonhemolytic streptococci.

TABLE 3. Numbers of swabs from which streptococci were isolated mixed with other organisms and from which streptococci only were isolated in three media

Medium	Total no. of swabs from which streptococci were isolated in mixed culture	No. of swabs from which streptococci were isolated in pure culture (% of total)	Total no. of positive cultures
Blood agar	119	7 (3.7)	126
CNA	87	99 (53)	186
COBA	5	185 (97)	190

The inhibition on COBA medium of competing flora in the clinical specimens investigated is almost complete. All of the gram-negative organisms were inhibited. Of the competing gram-positive organisms, three strains of *Staphylococcus* spp. and two coryneform strains grew on COBA medium. Although staphylococci are expected to be susceptible to oxolinic acid in concentrations lower than those used in COBA medium (17), resistant strains do occur. It is inevitable, given the ability of bacteria to develop resistance to antibiotics, that bacteria resistant to antibiotics chosen to suppress them in culture media will be encountered.

COBA medium was compared with CNA medium because neither colistin nor nalidixic acid inhibits the growth of streptococci (Petts, M.S. thesis, University of Surrey, Guildford, Surrey, England), and CNA is well established as a medium for the isolation of gram-positive cocci. Gram-negative organisms were also completely inhibited on CNA medium but staphylococci and coryneforms grew well. Altogether, 47% of the swabs from which streptococci were isolated on CNA medium also grew staphylococci or coryneforms or both. In approximately one-third of these specimens, the growth of the competing flora was sufficiently heavy to mask the streptococcal colonies. As isolated colonies were not available for subculturing, this masking produced delays in identification and susceptibility testing. This was a disadvantage of CNA medium particularly when the staphylococcal colonies considerably outnumbered the streptococcal colonies. Similar problems have been reported by other workers (6). Overgrowth of streptococci by other organisms did not occur on COBA medium, and subculturing for further testing was always possible from the original plate.

When the streptococcal isolation rates on COBA and CNA media were compared with those on Columbia blood agar, there was a significant difference in the results: for blood agar and COBA, $X^2 = 24.78$ and $P < 0.01$; for blood agar and CNA, $X^2 = 22.52$ and $P < 0.01$. There was no significant difference in the isolation of streptococci on COBA and CNA media ($X^2 = 0.074$ and $P > 0.95$).

COBA medium appears to have several advantages over other previously described selective media for streptococci. Neither colistin nor oxolinic acid at the concentrations used appear to have adverse effects on the growth of streptococci. Agents previously described to inhibit gram-positive competing organisms can be shown to have severe effects on

streptococci even at subminimal inhibitory concentrations. This is particularly true of gentamicin (6, 11, 14, 16, 19), amikacin, fucidic acid (9), neomycin (20), and sulfamethoxazole-trimethoprim (3). Adverse effects have also been noted for crystal violet and sodium azide. Both colistin and oxolinic acid are thermostable and can be stored at high ambient temperatures without deterioration.

Not only are high rates of isolation achieved with COBA medium but, as pure growths are usually obtained, early identification of the organisms is possible. The medium therefore will be of particular value in situations where streptococci are pathogens and reliable isolation and early identification is important.

LITERATURE CITED

1. **Blanchette, L. P., and C. M. S. Lawrence.** 1967. Group A streptococcus screening with neomycin blood agar. Am. J. Clin. Pathol. **37**:441–443.
2. **Dilworth, J. A., P. Stewart, J. M. Gwaltney, J. O. Hendley, Jr., and M. A. Sande.** 1975. Methods to improve detection of pneumococci in respiratory secretions. J. Clin. Microbiol. **2**:453–455.
3. **Dykstra, M. A., J. C. McLaughlin, and R. C. Bartlett.** 1979. Comparison of media and techniques for detection of group A streptococci in throat swab specimens. J. Clin. Microbiol. **9**:236–238.
4. **Ellner, P. D., C. J. Stoessel, E. Drakeford, and F. Vassi.** 1966. A new culture medium for medical bacteriology. Am. J. Clin. Pathol. **45**:502–504.
5. **Freeburg, P. W., and J. M. Buckingham.** 1976. Evaluation of the bacti-lab streptococci culture systems for selective recovery and identification of group A streptococci. J. Clin. Microbiol. **3**:443–448.
6. **Gray, B. M., M. A. Pass, and M. C. Dillon, Jr.** 1979. Laboratory and field evaluation of selective media for isolation of group B streptococci. J. Clin. Microbiol. **9**:466–470.
7. **Gunn, B. A., D. K. Ohashi, C. A. Gaydos, and E. A. Holt.** 1977. Selective and enhanced recovery of group A and B streptococci from throat cultures with sheep blood agar containing sulfamethoxazole and trimethoprim. J. Clin. Microbiol. **5**:650–655.
8. **Islam, A. K. M. S.** 1977. Rapid recognition of group B streptococci. Lancet **i**:256–257.
9. **Lowbury, E. J. L., A. Kidson, and H. A. Lilly.** 1964. A new selective blood agar medium for *Streptococcus pyogenes* and other haemolytic streptococci. J. Clin. Pathol. **17**:231–235.
10. **Master, P. L., W. Brunfitt, R. L. Mendez, and M. Likar.** 1952. Bacterial flora of the upper respiratory tract in Paddington families. Br. Med. J. **4**:1200–1205.
11. **Milatovic, D.** 1981. Comparison of five selective media for beta-haemolytic streptococci. J. Clin. Pathol. **34**:556–558.
12. **Murray, P. R., A. D. Wold, C. A. Schreck, and J. A. Washington II.** 1976. Effects of selective media and atmosphere of incubation on the isolation of group A streptococci. J. Clin. Microbiol. **4**:54–56.
13. **Nakamizo, Y., and M. Sato.** 1982. A new selective media for the isolation of *Streptococcus haemolyticus*. Am. J. Clin. Pathol. **57**:228–235.
14. **Nichols, T., and R. Freeman.** 1980. A new selective media for *Streptococcus pneumoniae*. J. Clin. Pathol. **33**:770–773.
15. **Schmid, R. E., J. A. Washington II, and J. P. Anhalt.** 1978. Gentamicin blood agar for isolation of *Streptococcus pneumoniae* from respiratory secretions. J. Clin. Microbiol. **7**:426–427.
16. **Sondag, J. E., R. K. Morgens, J. E. Hoppe, and J. J. Marr.** 1977. Detection of pneumococci in respiratory secretions. Clinical evaluation of gentamicin blood agar. J. Clin. Microbiol. **5**:397–400.
17. **Turner, F. J., S. M. Ringel, J. F. Martin, P. J. Storino, J. M. Daly, and B. S. Schwartz.** 1967. Oxolinic acid, a new synthetic antimicrobial agent, p. 475–479. Antimicrob. Agents Chemother. 1967.
18. **Vincent, W. F., W. E. Gibbons, and H. A. Gaafar.** 1971. Selective medium for the isolation of streptococci from clinical specimens. Appl. Microbiol. **22**:942–943.
19. **Waitkins, S. A.** 1982. A selective and differential medium for group B streptococci. Med. Lab. Sci. **39**:185–188.
20. **Wren, M. W. D.** 1980. Multiple selective media for the isolation of anaerobic bacteria from clinical specimens. J. Clin. Pathol. **33**:61–65.